A NOVEL SPECTROSCOPIC METHODOLOGY FOR THE INVESTIGATION OF INDIVIDUAL *BACILLUS* SPORES

Troy A. Alexander*, Paul Pellegrino and James B. Gillespie
U.S. Army Research Laboratory, AMSRL-SE-EO, 2800 Powder Mill Road, Adelphi, MD 20783

ABSTRACT

A methodology has been developed for the investigation of bacterial spores. Specifically, this method has been used to probe the spore coat composition of two different Bacillus stearothermophilus variants. This unique detection scheme is based on the NIR Surface-Enhanced-Raman-Scattering (SERS) from single, optically trapped, bacterial spores. The light from a 787-nm laser diode was used to trap/manipulate as well as simultaneously excite the SERS of an individual bacterial spore. Comparison of normal Raman and SERS spectra reveal not only an enhancement of the normal Raman spectral features but also the appearance of spectral features absent in the normal Raman spectrum.

INTRODUCTION

Since the seminal work of Ashkin in 1970, based on a dual laser beam system[1] and later work in 1986 employing a single laser beam apparatus[2], the laser tweezer phenomenon has been broadly accepted as a powerful tool to study viruses[3], vegetative bacterial cells[4-8], mammalian cells[9] and colloidal crystallization in microgravity environments[10]. More recently, this technique in combination with various Raman detection schemes has been applied to the investigation of inorganic gas bubbles[11], aerosols[12], emulsion particle polymerization[13], liquid-liquid extraction of toluene in water[14], organic nanoparticles[15], yeast cells[8] and solid-phase peptide synthesis[16]. Although significant work has been devoted to using this unique tool to study vegetative bacterial cells, to our knowledge there have been no published reports that employ optical trapping to study bacterial spores.

Raman spectroscopy has been an invaluable technique in the study of various chemical systems and has become widely accepted as an analytical characterization methodology[17,18]. The attractiveness of this technique stems from 1) its narrow spectral band structure, 2) its lack of interference from water and 3) its relative insensitivity to the excitation wavelength employed. However, unenhanced Raman spectral features are considered to be relatively weak thus requiring relatively lengthy collection times. In many applications, high quality spectra may be achieved with shortened acquisition times and improved spectral features by exploiting various amplification techniques. Namely, Resonance Raman Scattering (RRS), Surface Enhanced Resonance Raman Scattering (SERRS) or Surface-Enhanced-Raman-Scattering (SERS).[17-19] RRS and SERRS are typically conducted using ultraviolet excitation to facilitate electronic excitation (i.e. strong absorption) in the analyte of interest. This precludes their implementation in a single beam optical trapping configuration since the trapped particle experiences MW/cm^2 intensity levels. Further, UV intensities of this magnitude coupled with significant UV-light absorption would photdecompose most analytes. In contrast, SERS is well suited for combination with the optical trapping

* Author to whom correspondence should be sent.

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01 OCT 2005	**N/A**	**-**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
A Novel Spectroscopic Methodology For The Investigation Of Individual Bacillus Spores	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **U.S. Army Research Laboratory, AMSRL-SE-EO, 2800 Powder Mill Road, Adelphi, MD 20783**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES
See also ADM001851, Proceedings of the 2003 Joint Service Scientific Conference on Chemical & Biological Defense Research, 17-20 November 2003. , The original document contains color images.

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**UU**	**7**	

phenomenon since this technique may be performed using near-infrared excitation. Moreover, this is attractive since most analytes do not absorb strongly in the NIR.

Currently, the mechanism of the SERS effect is not fully understood; however, a plausible explanation for at least a significant portion of the spectral amplification has been attributed to an increase in the electromagnetic field strength encountered by the analyte. Additionally, with certain metal-analyte systems enhancements from 4 to 14 orders magnitude are readily achieved[17]. Since early reports on this technique in the late 1970's, its acceptance has not been as widespread as expected. This limited acceptance is in large part due to the lack of fabrication reproducibility in most SERS substrates. Relatively recent advances in SERS-active substrate fabrication have addressed this reproducibility issue. Specifically, these substrates are easily wavelength adjustable, durable, biocompatible and possess a long shelf-life.[19]

We report here, to our knowledge, the first simultaneous exploitation of the optical trapping and Surface-Enhanced-Raman-Scattering phenomena to detect a single bacterial spore. Further, a method based on their tandem application has been used to detect and discriminate two different *Bacillus stearothermophilus* variants; namely, *Bacillus stearothermophilus* (ATCC 10149) and *Bacillus stearothermophilus* (ATCC 7953). In addition, we describe a near-infrared Raman Tweezer Detection System (NIR-RTDS) that may be used to detect single, optically trapped biologically important particles (i.e., bacterial spores) as well as solutions occupying the trapping volume. Preliminary Raman and SERS spectra measured using this method have been examined for uniqueness and the applicability of these spectra for the discrimination of *Bacillus stearothermophilus* spores at the strain level.

EXPERIMENTAL

Instrumentation.

As shown in Figure 1, the near-infrared Raman Tweezer Detection System (NIR-RTDS) used in this study is similar in concept to those described previously.[9,11-12,14-16] As illustrated, 787-nm light from a commercially available temperature controlled, laser diode (**LD**, Thor Labs) capable of 50-mW output power was collimated through a Faraday optical isolator (**OI**; Electro-Optics Technology, Inc; Model # LD38I780) and a circularizing anamorphic prism pair (**PP**, Thor Labs) before passing through an interference filter (**IF**; Omega Optical; p/n 785DF10) with better than 70% transmission at 785-nm. The circular beam was then reflected by a holographic SuperNotch-Plus^{TM} filter (**SNF**; Kaiser Optical Systems, Inc.; p/n HSPF-785.0AR-1.0) centered at 785.0-nm into a Nikon TMS inverted microscope. A 50-mm focal length lens (**FL**; Thor Labs, p/n LA1131-B) was placed before the microscope to expand the laser beam and efficiently fill the back aperture of the focusing objective. This expanded beam was then focused by a 100X oil immersion microscope objective (**100X OBJ**; Nikon TE 300, N. A. 1.25) into the sample with a spot size of 1 – 2 μm at the focus (with an intensity of ~0.5 MW/cm^2 at the beam focus). A dichroic mirror (**DM**) was placed inside of the microscope housing, before the sample to deflect the laser light into the microscope objective. The position of the sample at the microscope stage was controlled in three dimensions using piezoelectric-driven actuators (Model 8302; New Focus, Inc.) having a minimum linear motion of less than 30-nm.

Both Rayleigh and inelastic scattered light from a single, trapped particle was collected by the same 100X oil immersion objective and collimated onto and directed by the dichroic mirror through two SNF's with optical densities better than 6.0 (to remove most of the Rayleigh scattered light) onto the entrance slit of a 0.275-m polychromator (SpectraPro®-275, Acton Research Corporation) and liquid nitrogen-cooled, back illuminated CCD (Spec-10:100BR, Roper Scientific) for spectral analysis. Light coupled into the polychromator was laterally dispersed across the 1340 X 100 pixel CCD chip by a 1200 groove/mm grating blazed at 750-nm. The polychromator was controlled through a digital-to-analog converter (**D/A**) and data was collected from the charge-coupled-device (**CCD**) by a personal computer though a 16-bit interface card using the WinSpec/32^{TM} software package (Version 2.5.8.1, Roper Scientific). To increase the S/N ratio the output of the CCD was vertically binned prior to readout. The area immediately surrounding the optical trap in the sample was imaged through the microscope objective and imaging optics (Edmund Industrial Optics) by a NIR-sensitive video camera (NEC TI-24A). A reflective neutral density filter (**ND**) was

placed before the camera to further reduce the amount of laser light reaching the camera. Images from this video camera were displayed on a video monitor for observation and digitized for later inspection using an 8-bit analog-to-digital (**A/D**) framegrabber (PixelSmart, Lewiston NY). All spectra were collected with a laser excitation power of 20-mW (before the microscope), 500-ms integration, 100 co-additions at a spectral resolution of 7.5 cm^{-1} unless otherwise stated.

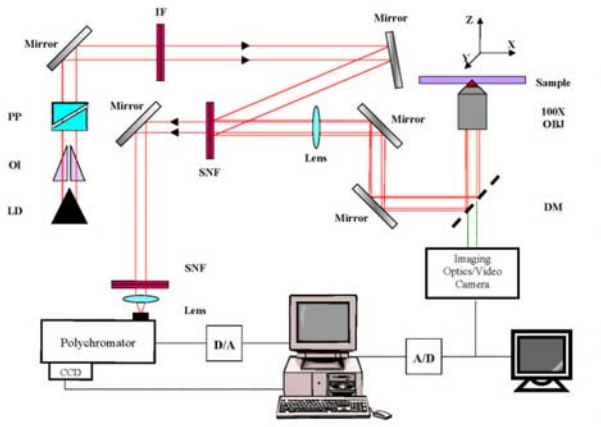

Figure 1. Schematic diagram of Near-infrared Raman-Tweezer Detection System (NIR-RTDS). See text for description.

SERS-substrate fabrication.

SERS-active substrates were prepared by a method similar to that suggested by Grabar, Freeman, Hommer and Natan[19] using 60-nm gold colloids synthesized in-house. Au colloids of 60-nm size were freshly prepared by a previously published[20] method were coupled to a silanized substrate by immersing the washed glass in the aqueous colloidal suspension for 24-h at room temperature. Subsequent to the colloidal coupling the SERS substrates were washed with and stored in deionized water until needed for analysis. UV-visible absorbance measurements (not shown) of these substrates reveal a Surface Plasmon Resonance (SPR) optimally excited with 715 to 775-nm light. SERS-substrates fabricated through this method may be near optimally excited using the NIR-RTDS.

RESULTS AND DISCUSSION

Bacillus stearothermophilus (ATCC 10149 and ATCC 7953), the two *Bacillus* spore strains selected for this study, were chosen for their taxonomic closeness to each other. Further, the genetic similarity between these two variants pose a challenge in their determination since they are expected to be similar in shape, size and immunoassay reactivity[21].

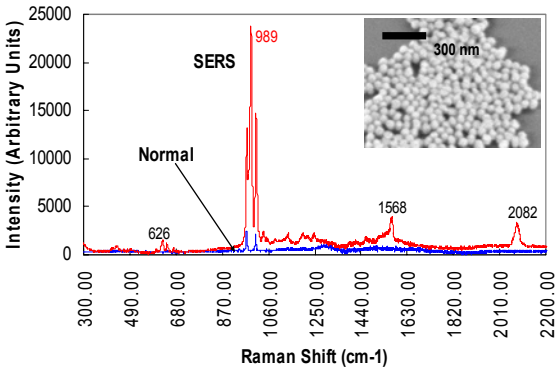

Figure 2. Normal raman and Surface-Enhanced-Raman-Scattering (SERS) spectra of pyridine taken using NIR-RTDS. Inset shows SEM image of 60-nm gold colloids immobilized on glass.

The SERS-activity of a representative immobilized Au colloid substrate was ascertained by measuring the spectrum of neat pyridine at an immobilized colloidal mass and subsequently measuring the same neat pyridine solution in a vacant region of the substrate. As illustrated in Figure 2, the SERS spectrum shows the appearance of new bands at 626, 989, 1568 and 2082 cm^{-1} which are not present in the un-enhanced pyridine Raman spectrum. The central band in the ring breathing region at 989 cm^{-1} is especially interesting since it has been reported to be indicative of Surface-Enhancement of pyridine[22]. That is, the central band in this triplet is present in the pyridine SERS spectrum but not the pyridine normal Raman spectrum. Therefore, the appearance of the 989 cm^{-1} is a good indicator of a substrate's SERS activity. Moreover, the appearance of the band in the pyridine SERS spectrum verifies that the fabricated substrates are in fact SERS-active. Depicted in the inset of this figure is a SEM image of a Au colloid mass immobilized on glass. The individual gold colloid particles composing the mass are apparent in the image

SERS Spectra of Single, Optically Trapped Bacterial Spore.

Figure 3A illustrates a schematic diagram of the method used to measure the SERS spectrum of a single bacterial spore. As shown, a three-dimensionally trapped spore is captured and vertically translated towards a gold colloid-coated microscope slide. NIR light transmitted through and around the spore subsequently excites the downward-directed Surface Plasmon Resonance (SPR) generated electric field of an adjacent immobilized colloidal mass. In fact, the amplified electric field is expected to only influence constituents of the spore coat and not the entire spore. That is, this methodology can be used to selectively probe the spore coat. This is attractive since it facilitates the isolation and study of bacterial spore coat constituents through non-physical means. In addition, the unique spore isolation capability inherent in optical trapping facilitates studying the same bacterial spore in different environments (i.e. normal Raman versus SERS conditions). This allows the investigation of Bacillus spores while eliminating spore-to-spore composition variability.

Depicted in Figure 3B are the normal Raman and SERS spectra for the same optically trapped *Bacillus stearothermophilus* (ATCC 10149) spore. These spectra are dominated by bands at 1005, 1345, 1434 and 1553 and 1005, 1135, 1246, 1360, 1675 and 1860 cm^{-1} in the normal Raman and SERS spectra, respectively. Further, the SERS spectrum is enhanced by approximately two orders of magnitude over the normal spectrum. This enhancement is admittedly smaller than those previously reported for various analyte-metal systems (typically, 4 to 14 orders). However, it should be kept in mind that these enhancements were achieved in analyte-substrate systems where the enhancement is due to a combination of 1) a substantially intensified electric field and 2) a charge transfer (i.e. chemical) mechanism. Further, in the current scheme (Figure 3A), the latter enhancement mechanism is most probably inactive since the optically trapped spore is well separated (tens of nanometers) from the enhancing feature and does not facilitate charge transfer between the trapped particle and metal surface. The complexity of these samples (i.e. bacterial spores) makes it difficult if not impossible to make band assignments. The only exception being the dipicolinic acid (DPA) band in the "ring-breathing" region. Specifically, the common band in these spectra at 1005 cm^{-1} is most likely due to dipicolinic acid (DPA), a major constituent of bacterial spores. A comparison of the SERS and normal spectra illustrate that the far-Stokes shifted bands in the SERS spectrum are enhanced more than the DPA ring breathing band at 1005 cm^{-1}. This can be understood in light that the moieties responsible for these spectral features most probably experience a stronger electric field than DPA. Since the DPA band and the far-Stokes shifted bands in the normal (i.e., whole spore) spectrum are comparable in intensity (i.e., Raman cross-section); this implies that the far-Stokes shifted constituents are held closer to the spore surface than DPA.

Plotted in Figure 4A are representative SERS spectra for two different optically trapped *Bacillus stearothermophilus* (ATCC 10149) spores. Both spectra are dominated by bands at 1005, 1360, 1675 and 2092 cm^{-1} as well as a pronounced shoulder at 1860 cm^{-1}. In general, the shape of these spectra are similar; however, distinct bands at 1140, 1195, 1245 and 1537 cm^{-1} are present in the spectrum labeled **B** but absent in the **A** spectrum. Additionally, the band intensity in the **B** spectrum at 1360 cm^{-1} is much smaller than this band's intensity in the **A** spectrum. These intensity differences as well as the appearance of pronounced bands in spectrum **B** in the spectral region from 1060 to 1250 cm^{-1} infer that the distribution

and/or relative concentration of Raman-active analytes are different in spore **A** and spore **B**. That is, the analytes responsible for the bands between 1060 and 1250 cm^{-1} in spectrum **B** are held closer to the spore surface and penetrate deeper within the SPR-generated electric field in spore **B** than in spore **A**. Further, the pronounced appearance of these bands in spectrum **B** illustrates the biodiversity present in this spore population.

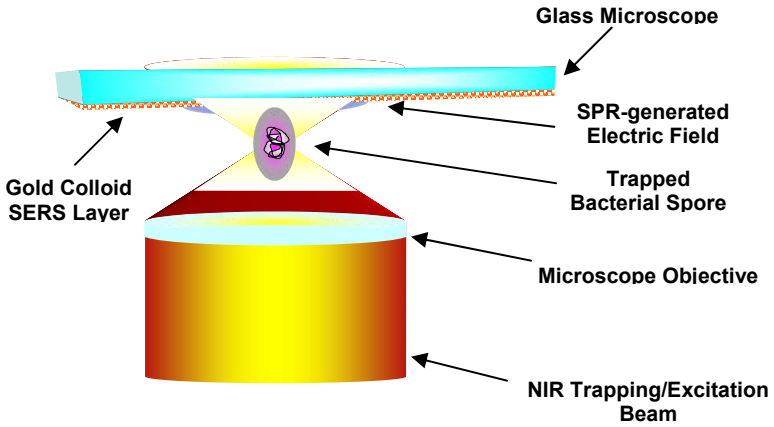

Figure 3A. Schematic diagram of method used to measure SERS spectrum of single, optically trapped bacterial spore.

Shown in Figure 4B are SERS spectra representing two different *Bacillus stearothermophilus* (ATCC 7953) spores. These spectra are constituted by pronounced bands at 585, 995, 1348 and 1668. Although, the plotted spectra are similar in shape over the measured spectral range (300 to 2200 cm^{-1}), large intensity variations are obvious between spectra **A** and **B** especially in the short-Stokes shifted (from 300 to about 1300 cm^{-1}) spectral features. Additionally, a strong band at 1575 cm^{-1} is present in spectrum **A** but absent in spectrum **B**. Further, a band in the "ring-breathing" region at 995 cm^{-1} which is attributed to dipicolinic acid is blue shifted 10 cm^{-1} from the spectra in Figure 4A. This shift infers that dipicolinic acid occurs in a slightly different chemical environment in the two different *Bacillus stearothermophilus* strains (namely, ATCC 10149 and ATCC 7953) studied. Moreover, the pronounced band at 585 cm^{-1} as well as the DPA band shift around 1000 cm^{-1} implies that the composition of *Bacillus stearothermophilus* (ATCC 7953) is significantly different than that of *Bacillus stearothermophilus* (ATCC 10149).

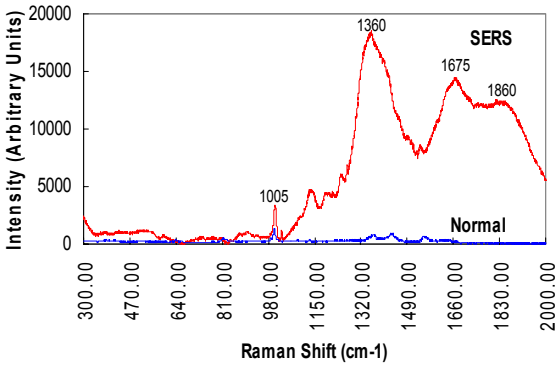

Figure 3B. Normal Raman and Surface-Enhanced-Raman-Scattering (SERS) spectra of single, optically trapped *Bacillus stearothermophilus* (ATCC 10149) spore.

The SERS spectral differences between the *Bacillus stearothermophilus* spores studied are more apparent in Figure 5. Shown in this figure are averaged SERS spectra for *Bacillus stearothermophilus* (ATCC 7953) and *Bacillus stearothermophilus* (ATCC 10149) intensity normalized at 1348 cm^{-1}. As illustrated, these spectra have common features near 1348, 1670 and 1860 cm^{-1}. However, more importantly the ATCC 7953 spectrum is distinguishable from ATCC 10149 by pronounced bands at 585

and 1576 cm^{-1}. In addition, the ATCC 10149 spectrum shows a pronounced band near 2100 cm^{-1} which is less obvious in the 7953 spectrum. Further, inspection of the "ring-breathing" region (near 1000 cm^{-1}) demonstrates a shift from 995 cm^{-1} in the ATCC 7953 spectrum to 1005 cm^{-1} in the ATCC 10149 spectrum. These data show great potential for using the optical trapping and SERS phenomena, in tandem, to facilitate the strain-level discrimination of *Bacillus stearothermophilus* spores.

CONCLUSIONS

In summary, a near-infrared Raman-Tweezer Detection System (NIR RTDS) has been constructed and applied to the development of a novel methodology for the detection of single, optically trapped *Bacillus* spores. This method is based on the simultaneous exploitation of the optical trapping and SERS phenomena. Two different *Bacillus stearothermophilus* spore strains have been studied using this methodology. Results show this method facilitates detection of SERS spectrum from a single, optically trapped bacterial spore. Further, spectral features measured using this method, demonstrate it may be possible to discriminate between the two *Bacillus stearothermophilus* strains studied.

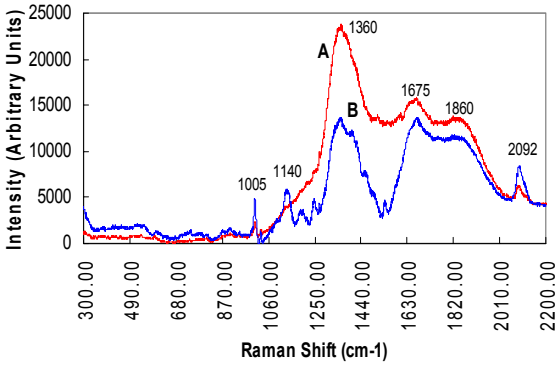

Figure 4A. Surface-Enhanced-Raman-Scattering (SERS) spectra of two different single, optically trapped *Bacillus stearothermophilus* (ATCC 10149) spores.

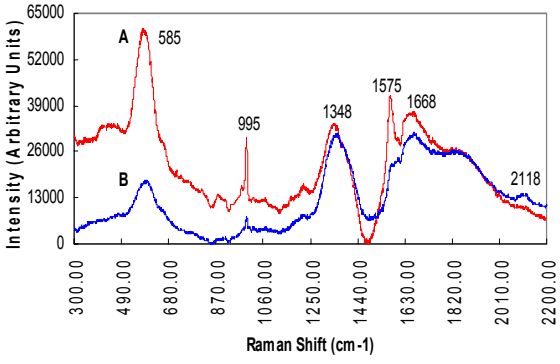

Figure 4B. Surface-Enhanced-Raman-Scattering (SERS) spectra of two different single, optically trapped *Bacillus stearothermophilus* (ATCC 7953) spores.

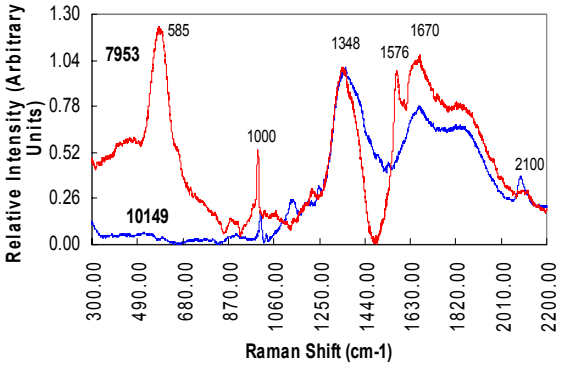

Figure 5. Intensity normalized Surface-Enhanced-Raman-Scattering (SERS) spectra of single, optically trapped *Bacillus stearothermophilus* (ATCC 10149) and *Bacillus stearothermophilus* (ATCC 7953) spores.

REFERENCES

1. A. Ashkin, *Physical Review Letters*, **24**, 156 – 159, 1970.
2. A. Ashkin, J. M. Dziedzic, J. E. Bjorkholm, and S. Chu, *Optics Letters*, **11**, 288 – 290, 1986.
3. A. Ashkin and J. M. Dziedzic, *Science*, **235**, 1517 – 1520, 1987.
4. A. Ashkin, J. M. Dziedzic, and T. Yamane, *Nature*, **320**, 769 – 771, 1987.
5. A. Ashkin, K. Schutze, J. M. Dziedzic, U. Euteneuer and M. Schliwa, *Nature*, **348**, 346 – 348, 1990.
6. W. H. Wright, G. J. Sonek, Y. Tadir, and M. W. Berns, *IEEE Journal of Quantum Electronics*, **26**, 2148 – 2157, 1990.
7. M. Ericsson, D. Hanstorp, P. Hagberg, J. Enger, and T. Nystrom , *Journal of Bacteriology*, **182**, 5551 – 5555, 2000.
8. C. Xie, M. A. Dinno, and Y. Li, *Optics Letters*, **27**, 249 – 251, 2002.
9. J. Guck, R. Ananthakrishnan, H. Mahmood, T. J. Moon, C. C. Cunningham, and J. Kas, *Biophysical Journal*, **81**, 767 – 784, 2001.
10. A. Resnick, *Review of Scientific Instruments*, **72**, 4059 – 4065, 2001.
11. M. Lankers, J. Popp, G. Rossling, and W. Kiefer, *Chemical Physics Letters*, **277**, 331 – 334, 1997.
12. J. F. Lubben, C. Mund, B. Schrader, and R. Zellner, *Journal of Molecular Structure*, **480 - 481**, 311 – 316, 1999.
13. V. E. Roman, J. Popp, E. Urlaub, S. Schlucker, M. Lankers, G. Robling, and W. Kiefer, *Journal of Molecular Structure*, **482 - 483**, 497 – 501, 1999.
14. K. Ajito, M. Morita, and K. Torimitsu, *Analytical Chemistry*, **72**, 4721 – 4725, 2000.
15. K. Ajito and K. Torimitsu, *Applied Spectroscopy*, **56**, 541 – 544, 2002.
16. M. P. Houlne, C. M. Sjorstrom, R. H. Uibel, J. A. Kleimeyer, and J. M. Harris, *Analytical Chemistry*, **74**, 4311 – 4725, 2002.
17. J. R. Ferraro and K. Nakamoto, *Introductory Raman Spectroscopy*, Chapter 3, Academic Press, San Diego and London, 1994.
18. D. L. Gerrard, "Organic and Petrochemical Applications of Raman Spectroscopy", *Analytical Raman Spectroscopy*, J. G. Grasselli and B. J. Bulkin, 301 - 304, John Wiley and Sons, Inc., New York, 1991.
19. K. Grabar, R. G. Freeman, M. B. Hommer, and M. J. Natan, *Analytical Chemistry*, **67**, 735 – 743, 1995.
20. L. G. Olson, Y. S. Lo, T. P. Beebe, Jr and J. M. Harris, *Analytical Chemistry*, **73**, 4268 – 4276, 2001.
21. G. W. Gould and A. Hurst, *The Bacterial Spores*, Chapter 7, Academic Press, London and New York, 1969.
22. L. Dawei, W. Jian, X. Houwen, S. Xu and L. Fan-Chen, *Spectrochimica Acta*, **43A**, 379 - 382, 1987.